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Predictors of Acute Lung Injury

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14. ABSTRACT

This study measured inflammatory mediators in tracheal secretions and plasma of subjects with severe multiple blunt trauma for 4 days post injury. The goal was to determine if biomarkers in tracheal fluid could be used to discriminate between subjects who developed acute lung injury (ALI) after Day 1 from those who did not develop ALI. The tracheal samples were analyzed by enzyme-linked immunosorbent assay for quantities of tumor necrosis factor (TNF), interleukin 1 beta (IL-1β), IL-6, IL-8, IL-10, C-reactive protein, pentraxin 3 (PTX3), and activated complement 5 daily for 4 days. The diagnosis of ALI or acute respiratory distress syndrome was made using the international consensus criteria. Comparisons were made from samples collected on the first 24 hours following injury (Day1) between the No ALI group (n=15) and the ALI group (n=6). The levels of all eight mediators on Day 1 were higher in the No ALI group vs. levels in the group that developed ALI after Day 1. The levels of TNF, IL-1β, and PTX3 were statistically higher in the No ALI group. The significant differences in biomarkers in tracheal fluid demonstrate that these markers are potentially predictive of which trauma subjects are at risk for ALI.

15. SUBJECT TERMS
Blunt trauma, biomarkers, ARDS, ALI, cytokines, tracheal secretions

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1.0 SUMMARY

Twenty-six subjects with multiple trauma were studied to determine the potential for using biomarkers in tracheal aspirates to determine the potential as predictors of susceptibility for acute lung injury (ALI). Informed consent was obtained and tracheal fluid samples were collected from the supraglottic area and the main-stem bronchus along with plasma samples daily for four consecutive days. Eleven of the subjects developed ALI/acute respiratory distress syndrome (ARDS) during the study: five developed ARDS on baseline, Day 1 (B-ALI), and were discarded from further analysis. Potential for predictability was determined by statistically significant differences in biomarker levels between the subjects who developed ALI after Day 1 (D-ALI) and the subjects who did not develop ALI (N-ALI). All eight biomarkers [interleukin 1 beta (IL-1β), tumor necrosis factor (TNF), IL-6, IL-8, IL-10, C-reactive protein (CRP), pentraxin 3 (PTX3), and activated complement 5 (C5a)] were detected in the tracheal aspirates from both groups of subjects. All eight biomarkers were lower in the delayed ALI group in the tracheal aspirates, indicating a reduced inflammatory response to the severe trauma in the trachea of the D-ALI group. The levels of TNF, IL-1β, and PTX3 were statistically significantly lower in the D-ALI group. The biomarkers in the supraglottic tracheal samples and plasma samples did not show any consistent pattern of response nor significant differences between the groups of trauma patients. Cytokine levels in tracheal aspirates clearly have the potential to be used as a predictor of susceptibility to develop ALI in trauma patients.

2.0 INTRODUCTION

Twenty to 30% (1-3) of patients who have multiple traumatic injuries [Injury Severity Score (ISS)>19] develop non-hydrostatic pulmonary edema and a resultant significant decrease in pulmonary oxygen transfer known as acute lung injury. The need to be able to predict which trauma patient will develop ALI can be particularly crucial in triaging the military trauma patient. Transportation of a patient with significant decreases in pulmonary gas exchange could be life threatening. Being able to accurately predict the patients who are susceptible to developing ALI would alter how all trauma patients are medically managed.

The approach taken to predict which at-risk patients are susceptible to developing ALI has been to identify biochemical markers that will enhance the clinical risk factors (4). The ideal biomarker would have considerable predictive value, be directly related to the pathophysiology of ALI, be quantifiable in blood or fluid from the lungs, and be able to be measured rapidly. Many of the reports in the literature focus on predicting ALI or the more severe form, ARDS, in patients from all causes (5,6) including sepsis, smoke inhalation, shock, aspiration, trauma, multiple transfusions, brain injury, etc. The sources of the biomarkers under consideration come from either plasma or bronchoalveolar lavage (BAL) fluid. Markers found in BAL are thought to be more specific to the pathophysiology of the lung but require bronchoscopy to collect. Most of the studies evaluating the ability of various markers to predict ARDS have been retrospective and include all trauma patients regardless of injury severity.

The present study evaluated the inflammatory response to multiple trauma by quantifying several biomarkers in tracheal secretions. Inflammatory mediators have been studied in tracheal secretions in non-intubated subjects from carefully collected sputum samples (bronchiectasis, chronic bronchitis, cystic fibrosis, etc.). Earlier experiments in this laboratory demonstrated that inflammatory mediators could be measured in tracheal fluid from non-injured subjects and swine

(7,8). Tracheal secretions are easy and simple to obtain from normal care of the intubated trauma patient.

The present study was designed to evaluate the predictability of the development of ALI using inflammatory mediators in the tracheal secretions from subjects with multiple traumatic injuries. Two areas of the trachea were sampled: above the cuff of the endotracheal tube (ETT) (supraglottic) and distal to the tip of the ETT in the large airways. A "cytokine storm" of many different cytokines develops in the early phase of ALI or ARDS (4) that remains during the course of the disease. We monitored three functional groups of cytokines, as these markers have been widely reported to be involved with the development of ALI/ARDS. These groups were (1) pro-inflammatory cytokines (IL-1β, TNF, and IL-6), (2) chemotactic cytokines (IL-8 and C5a), and (3) the several anti-inflammatory cytokines (IL-10, PTX3, and CRP). Multiple markers in each group were chosen because it was unknown how the trachea would respond to the development of ALI.

We found that the rate of development of ALI/ARDS in our study population was 46% (11/26); five patients developed ALI/ARDS on Day 1. The primary focus of this study was to determine if there were predictable differences on Day 1 between subjects who later developed ALI from those who did not, so these five patients were excluded from further analysis. We observed a consistent inverse relationship between the concentrations of these biomarkers and the development of ALI/ARDS: higher cytokine levels in the tracheal fluid were associated with not developing ALI/ARDS during the 4 days following injury.

3.0 PROCEDURES, METHODS, AND ASSUMPTIONS

3.1 Procedures

3.1.1 Subject Recruitment. Subjects were recruited from the trauma surgery service of Saint Louis University Hospital from June 2011 to September 2012. Patients who were identified with sufficient blunt trauma that they were likely to be intubated for 4 days were considered for enrollment. The inclusion and exclusion criteria are as follows:

Inclusion criteria:

1. Either gender 18-65 years
2. Health status of ISS >1 (need for intubation)
3. Patients admitted following physical blunt trauma and require intubation likely to continue for 4 days
4. Ability to provide informed consent (or by spouse, relative, or legal guardian)
5. Ability to speak English

Exclusion Criteria

1. Patient declines participation
2. Age less than 18 or greater than 65 years
3. Difficulty or failure in placing ETT, requiring more than two attempts
4. Pregnancy
5. Incarceration

This study was approved and patient consent obtained according to the guidelines approved by the Institutional Review Boards of Saint Louis University, Saint Louis University Hospital, and the U.S. Air Force. Out of a total of 86 patients who were evaluated and processed, 26 were enrolled. All subjects were initially intubated with the Mallinckrodt™ TaperGuard™ Evac oral ETT (Covidien, Mansfield, MA), but no other alterations were made in the normal intensive care for these patients.

3.1.2. Sample Collection. All subjects were intubated with the TaperGuard Evac oral ETT, which permitted the collection of samples of secretions from the supraglottic area of the trachea via the port above the cuff. These samples of tracheal secretions were obtained daily. Collection of the supraglottic tracheal samples required infusing lavage fluid (normal saline) and aspirating the lavage through the same infusion line [referred to as tracheal lavage samples]. Sequentially with obtaining the tracheal lavage samples, samples of tracheal aspirates were collected from the normal suctioning used to maintain uncongested airways in these patients. Samples of tracheal lavage, tracheal aspirate, and plasma were collected within 24 hours of the occurrence of the trauma (Day 1 tracheal aspirate samples taken 16.8 hours post injury) and at approximately 24-hour intervals thereafter for a total of 4 days.

3.2 Methods

3.2.1 Sample Processing and Analysis. Both tracheal fluid samples were collected and placed on ice and transported to the laboratory for processing within 1 hour of collection. Plasma samples were obtained from excess samples collected for normal laboratory tests according to Institutional Review Board guidelines. The tracheal fluid specimens were transferred to 15-mL sterile conical centrifuge tubes and mixed with a transfer pipet. The volume of each sample was measured and 250 µL of fluid were removed for cell count and differentials. Differentials were determined from concentrated slides prepared using a cytocentrifuge (Shandon Cytospin 2, Thermo Electron Corp., Runcorn, England). The samples were centrifuged for 15 minutes at 1350 rpm at 4 °C. After centrifugation, the supernatant was removed if the samples separated, and the supernatant was frozen in aliquots of at 50-500 µL. In some of the more viscous samples, separation was not achieved, so the samples were remixed to try to ensure a homogeneous sample for freezing in aliquots of 50-500 µL.
Heparinized and ethylenediaminetetraacetic acid plasma were obtained from each subject on each of the 4 days. Plasma was frozen in aliquots of 50-500 µL.

Aliquots of tracheal fluid and plasma were thawed for assay by enzyme-linked immunosorbent assay (ELISA) and protein assay (BCA, Thermo Scientific, St. Louis, MO). ELISA assays (all for human proteins) performed were the following:

- C5a (OptEIA C5a ELISA Kit II, Beckton Dickinson, St. Louis, MO) with a range of 78-5000 pg/mL
- CRP (CRP ELISA kit, R&D Systems, Minneapolis, MN, where all the following kits were also purchased) with a range of 0.78 to 25 ng/mL
- PTX3 (PTX3/TSG-14 kit) with a range of 0.31 to 20 ng/mL
- IL-1β (IL-1β/IL-1F2 kit) with a range of 3.9 to 250 pg/mL
- IL-6 (IL-6 kit) with a range of 3.12 to 300 pg/mL
- IL-8 (CXCL8-IL-8 kit) with a range of 31.2 to 2000 pg/mL

- IL-10 (IL-10 kit) with a range of 7.8 to 5000 pg/mL
- TNF-α (TNF-α kit) with a range of 15.6 to 10,000 pg/mL

All assays were performed in duplicate (triplicate for total protein assays) according to the manufacturer's instructions, and all tracheal fluids and plasmas were diluted at least 1:10 in dilution buffer specific for the assay. Samples were repeated at higher dilutions as necessary until they could be read on the standard curve. The coefficient of variation for the repeated measures was 5% for these biomarker analyses.

3.2.2 ALI/ARDS Determination. Diagnosis of ALI and ARDS was carried out from the patient blood gas data and the calibrated ventilator FiO2 setting using the International Consensus Criteria: PaO2/FiO2 <300 for ALI and <200 for ARDS (9). Gas transfer calculations were used along with radiographic evidence of diffuse infiltrates and clinical indication of absence of pulmonary hypertension to make the diagnosis. All ALI/ARDS diagnoses were verified by the same physician (CF) for consistency of interpretation.

3.3 Assumptions

3.3.1 Power Analysis. Based upon previous studies in this laboratory (7,8) that provided estimates of variance of these data in non-trauma subjects, we projected that we would need 15 subjects with ALI/ARDS who would have a 50% difference in values to provide a reasonable chance to reach statistical significance. Review of the subject pool in the trauma intensive care unit indicated that we would have to enroll 100 subjects to obtain the 15-16 indicated by the power analysis. An interim review of the data indicated that we had a much higher rate of ALI than expected but we did not anticipate the high number of patients with ALI on Day 1.

3.3.2 Study Design. To determine the potential of a biomarker as a predictor of risk for development of ALI, the biomarker levels would have to be statistically significantly different on the baseline day for the subjects who would develop ALI on subsequent days and the subjects who would never develop ALI. Therefore, the subjects were placed into three groups based on their development of ALI/ARDS: Group 1—no development of ALI/ARDS for all 4 days of study (N-ALI); Group 2—development of ALI/ARDS during the baseline 24 hours (B-ALI); and Group 3—development of ALI/ARDS delayed later than Day 1 (D-ALI). Since the goal was to determine a biochemical marker that would predict the development of ALI based on Day 1 values, B-ALI subjects were removed from the analysis. The study design then called for comparison of the levels of biomarkers on Day 1 data for D-ALI compared to Day 1 values for N-ALI. If significant differences were found in a biomarker, testing of the predictive value of that biomarker would have to be carried out on a much larger sample of trauma subjects to determine the number of false positive and false negative subjects relative to a criterion value in a more representative sample of trauma patients.

3.3.3 Data Analysis. Statistical analyses were performed using IBM SPSS Statistics 20 and Graph Pad Instat3 (San Diego, CA) programs. Data comparisons were made using non-parametric methods. Significance was determined using the Mann-Whitney test with $p<0.05$ being considered statistically significant.

4.0 RESULTS

4.1 Patient Description

The patients enrolled in this study are described in Table 1. The mean age for all 26 subjects was 39 years; 81% were male, with 21 Caucasians, 5 African Americans, and 1 Hispanic. The injuries to these subjects involved motor vehicles (cars, trucks, motorcycles) in 24 of the 26 cases. There was no difference in the ISS between the groups that had ALI/ARDS and those that did not. However, the ISS for the subjects who developed ARDS on Day 1 tended to be higher than the ISS for the other subjects. Sixteen of the 26 patients had pulmonary contusions noted on their radiologic exams at admission. Based on clinical findings at admission, there was no discernible difference between the subjects who did and did not develop ALI/ARDS.

Table 1. Descriptive Characteristics of Subjects with Blunt Traumatic Injury

Characteristic	ALI (N=26)		D-ALI (N=6)		N-ALI (N=15)		p-value <.05[a]
	N	%	N	%	N	%	
Mean Age (yr)	39		38		36		NS
Gender:							
Male	21	80.8	5	83.7	11	73.3	NS
Female	5	19.2	1	16.7	4	26.7	
Race:							
Caucasian	21	80.8	5	83.7	11	73.3	
African American	5	19.2	1	16.7	4	26.7	NS
Ethnicity:							
Non-Hispanic	25	96.2	6	100	14	93.3	NS
Hispanic	1	3.8	0	0	1	6.7	
Mean Body Mass Index	29		34		26		NS
Mean ISS	29		25		28		NS
Pulmonary Contusions	16	62	5	83.7	8	53	NS
Rib Fractures	16	62	3	50	9	60	NS
Pelvic Fractures	2	7.7	1	16.7	1	6.7	NS
Femur Fractures	4	15.4	0	0	4	26.7	NS

[a]t-test for means, Fisher's exact test for categorical variables, p-value <.05

4.2 Neutrophils and Protein

One of the hallmarks of inflammation in a tissue is the influx of phagocytic polymorphonuclear leukocytes (PMNs) into the tissue, which is the case with ARDS (10). All tracheal fluid samples were assessed for extent of the influx of cells with primarily PMNs into the trachea. Previous experiments with non-injured patients (7) indicated that a baseline of 7% PMNs existed in the tracheal fluid immediately following intubation. All of the subjects in the study being reported here had greater than 90% PMNs in the samples on all days. The considerable increase in mucous production in the airway of these subjects presented problems with accurate assessment of the extent of PMN influx. The cell counts were inaccurate because large numbers of PMN were enmeshed in the mucus, thus rendering the cell counts useless. It is

clear that PMNs moved into the trachea in large numbers, but this approach to assessment of PMN influx did not provide any data that would predict who would develop ALI/ARDS.

Sample protein levels were measured in all of the tracheal fluid samples to ensure the samples obtained were measuring tracheal secretions. Normal procedures used to care for the intubated airway were used to obtain these samples. This occasionally involved adding saline to the sample line to be able to clear the airway; some of this saline was used in the assessment of the biomarker concentrations. In a few samples, primarily saline was collected (protein below 1 mg/mL), which resulted in eliminating data from these specimens from the analysis. This was the only reason for the elimination of any sample from the analysis.

4.3 Cytokine Biomarkers

All of the biomarkers consisting of the chemotaxins (C5a and IL-8), pro-inflammatory cytokines (IL-1β, TNF, and IL-6), and the anti-inflammatory regulators (PTX3, CRP, and IL-10) were found in significant quantities on Day 1 in the trachea of subjects following blunt trauma. The concentrations of these inflammatory mediators were all higher in the N-ALI subjects than in the D-ALI group (Table 2). The largest difference observed was for PTX3, which had an 11-fold difference between the groups. Three of these mediators were statistically significantly lower in the D-ALI group: IL-1β, TNF, and PTX3. The actual p-values are listed in Table 2. It is apparent that several other biomarkers are approaching significance. The distribution of the individual subjects is shown in Figures 1-3.

All Day 1 cytokines measured in tracheal fluid during the first 24 hours were greater in the N-ALI subjects than in the D-ALI subjects (Table 2). The ratios of the median values for the D-ALI subjects to the N-ALI subjects on Day 1 are 0.48 for IL-1β, 0.08 for TNF, 0.04 for PTX3, 0.37 for CRP, 0.32 for IL-8, 0.19 for IL-6, 0.71 for C5a, and 0.45 for IL-10. The between group differences were statistically significant for IL-1β (medians 11079 vs. 5314 pg/mL), TNF (medians 1934 vs. 161 pg/mL), and PTX3 (medians 184 vs. 8 pg/mL). Although not significantly different, the p-values for CRP and IL-8 in particular would be candidates for any multivariate analysis where the number of subjects would be larger.

The range of values for each of the statistically significant biomarkers is shown in Figures 1, 2 and 3. These figures show the amount of overlap of ranges for each of these three cytokines.

Using the individual data box and whisker plots in Figures 1, 2, and 3, it is possible to estimate a value for each cytokine below which a patient would be predicted to develop ALI/ARDS after Day 1. By choosing the median value of the biomarker for the ALI group and applying it to each group, there are two false negatives and three false positives for IL-1β and PTX3 for a correct prediction of 14 of 19 patients (74%). Using the same criterion for TNF results in one false negative and three false positives or 79% correct. In addition, there appears to be a clearer separation between the groups for TNF.

The cytokine concentrations from the supraglottic area of the trachea could provide insight into the response of another segment of the tracheal tissue to injuries caused by multiple trauma. The median with interquartile ranges (IQRs) for these cytokines is shown in Table 3. The tracheal fluid concentrations are generally less than observed in the lower trachea except for IL-6, which is about 20% higher in the N-ALI group. Again, all of the biomarkers but IL-10 are lower in the group that developed D-ALI, although the reductions are much less except for TNF

and CRP. The only cytokine to approach a significant difference between the groups is CRP, where p=0.09.

Table 2. Levels of Inflammatory Mediators in Tracheal Aspirates (Distal to ETT) on Day 1 Following Severe Blunt Trauma[a]

Mediator	IL-β (pg/mL)	TNF (pg/mL)	PTX3 (pg/mL)	CRP (mg/L)	IL-8 (ng/mL)	IL-6 (pg/mL)	C5a (ng/mL)	IL-10 (pg/mL)
N-ALI								
N	13	13	13	13	13	13	13	13
median	11079	1934	184	915	413	5710	68	66
IQR	3091-47533	34-4923	0-337	88-1155	22-490	85-9857	16-272	0-273
D-ALI								
N	6	6	6	6	6	6	6	6
median	5314[b]	161[b]	8[b]	341	131	1105	48	30
IQR	1525-8448	11-1441	2-34	66-714	367-1430	364-2461	22-112	13-100
p-value N-ALI vs. ALI	0.046	0.04	0.04	0.13	0.13	0.28	0.28	0.51

[a]Subjects who did not develop ALI/ARDS for 4 days were compared to those who developed ALI/ARDs on Days 2, 3, or 4. Comparisons were made using non-parametric methods. Mann-Whitney test used to determine significance.
[b]Denotes p-value <0.05.

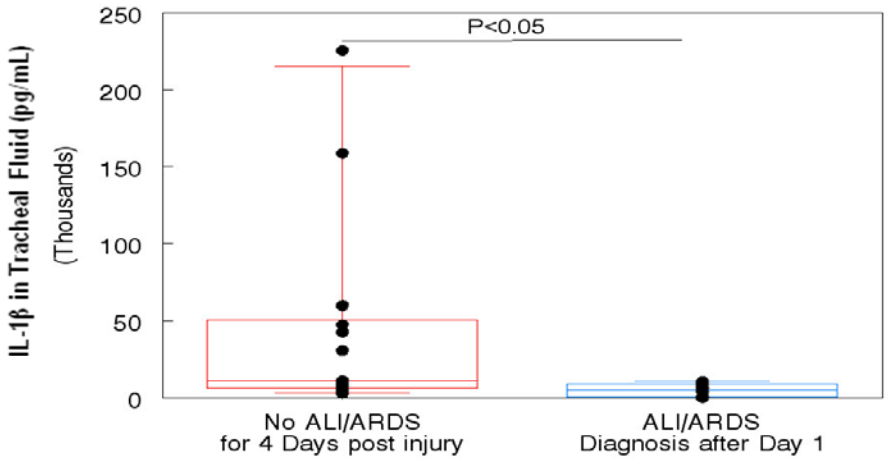

Figure 1. Concentrations of IL-1β for Each of the Subjects in the D-ALI and N-ALI Groups on Day 1 Following Blunt Trauma. *The N-ALI group had statistically significantly more IL-1β (n=13) than did the D-ALI group (n=6). Significance determined by Mann-Whitney test.*

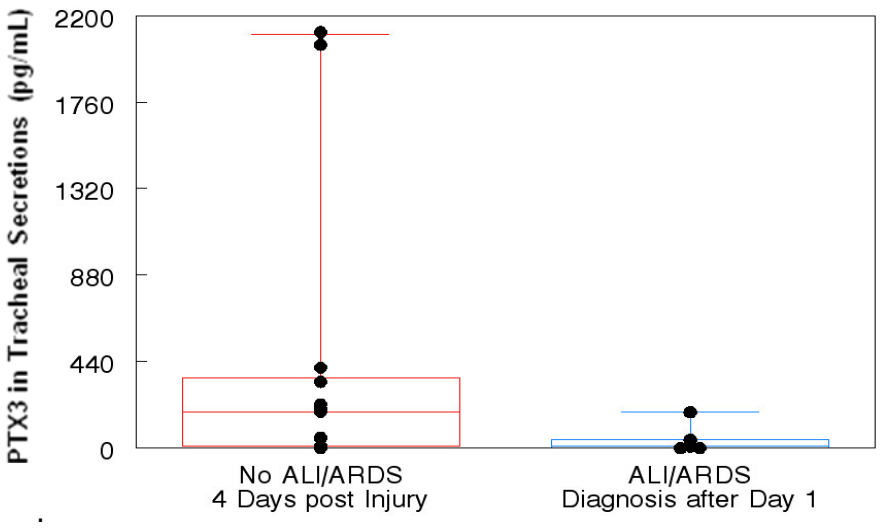

Figure 2. Concentrations of PTX3 for Each of the Subjects in the D-ALI and N-ALI Groups on Day 1 Following Blunt Trauma. *The N-ALI group had statistically significantly more PTX3 (n=13) than did the D-ALI group (n=6) (p<0.05). Significance determined by Mann-Whitney test.*

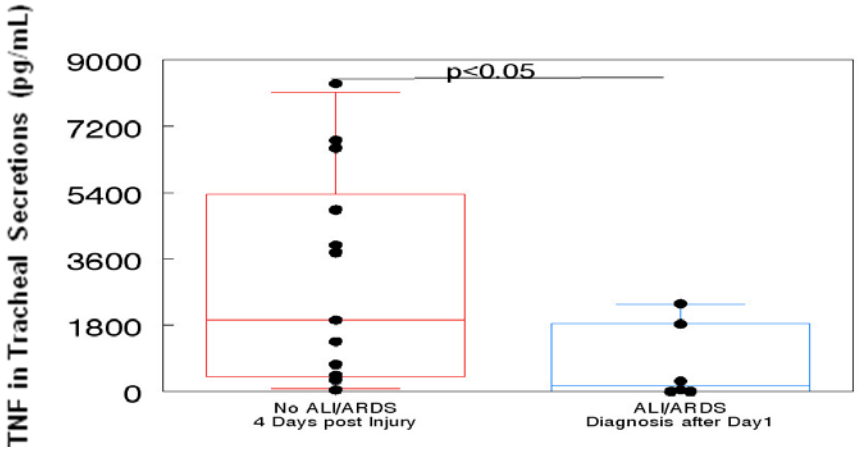

Figure 3. Concentrations of TNF for Each of the Subjects in the D-ALI and N-ALI Groups on Day 1 Following Blunt Trauma. *The N-ALI group had statistically significantly more TNF (n=13) than did the D-ALI group (n=6) (p<0.05). Significance determined by Mann-Whitney test.*

Table 3. Levels of Inflammatory Mediators in Tracheal Lavages Collected Above the Cuff of the ETT on Day 1 Following Severe Blunt Trauma[a]

Mediator	IL-β (pg/mL)	TNF (pg/mL)	PTX3 (pg/mL)	CRP (mg/L)	IL-8 (ng/mL)	IL-6 (pg/mL)	C5a (ng/mL)	IL-10 (pg/mL)
				N-ALI				
N	14	14	14	14	14	14	14	14
median	5022	22	22	821	20	7190	85	2
IQR	1635-8236	0-244	5-83	123-1738	14-73	898-11816	44-143	0-33
				D-ALI				
N	6	6	6	6	6	6	6	6
median	1958	0	11	104	14	2719	73	281
IQR	424-9805	0-323	2-32	66-332	6424-67142	385-8264	5-159	61-518
p-value N-ALI vs. ALI	0.60	0.90	0.39	0.09	0.49	0.41	0.78	0.90

[a]p-values for significance determined by Mann-Whitney test.

The concentrations of the biomarkers in plasma are shown in Table 4. The plasma samples were collected at the same time as the tracheal samples. There are two items worth noting: neither TNF nor IL-8 was detectable in these plasma samples, and the levels of CRP are very high as would be expected in blood from trauma patients (11). There were no statistical differences between the D-ALI and N-ALI groups, nor were the values consistently lower in the D-ALI group as they were in the trachea below the ETT.

Table 4. Levels of Inflammatory Mediators in Plasma Samples Collected on Day 1 Following Severe Blunt Trauma[a]

Mediator	IL-β (pg/mL)	TNF (pg/mL)	PTX3 (pg/mL)	CRP (mg/L)	IL-8 (ng/mL)	IL-6 (pg/mL)	C5a (ng/mL)	IL-10 (pg/mL)
				N-ALI				
N	15	b	15	15	b	15	15	15
Median	0		7	12400		520	26	42
IQR	0-0		4-11	4394-40771		337-1355	22-38	24-165
				D-ALI				
N	6		6	6		6	6	6
Median	0		32	7450		322	32	48
IQR	0-125		3-17	4879-25396		186-578	30-40	11-87
p-value N-ALI vs. ALI	0.55		0.64	0.91		0.30	0.53	0.78

[a]p-values for significance determined by Mann-Whitney test.
[b]Undetectable.

5.0 DISCUSSION

The ability to predict if a trauma patient is likely to develop ALI would play a significant role in triage in the military setting. Predictability of patients who are at risk for developing ALI/ARDS would also have many advantages in the clinical care of all trauma patients. It could lead to more aggressive treatment early on in the care of the patient, lead to improved understanding of the pathogenesis of ALI/ARDS, and could provide experimental therapies that are focused on biomarkers used as predictors (12). Predictive models have taken one of two paths: either the use of descriptive clinical measures (5,13) or the use of relevant biochemical markers in blood or alveolar fluid (2,12,14-16). Many of these predictive models have been adopted for use with trauma patients. The present investigation has used the latter approach

toward identifying biomarkers that could be used to identify trauma patients who are susceptible for developing ALI.

The weaker production of all of the inflammatory mediators on Day 1 by the subjects who eventually developed ALI/ARDS suggests that the trachea in these patients could not mount an adequate immune response to the trauma. This generalized response in the trachea is in contrast to the imbalance of inflammatory agents observed in the alveoli. Donnelly et al. (17) measured inflammatory mediators in bronchoalveolar lavage of patients with ARDS due to either sepsis or trauma (ISS of 33) within 24 hours of diagnosis. They reported that non-survivors had significantly lower levels of the anti-inflammatory agents IL-10 and IL-1β receptor antagonist than did the survivors. Donnelly interpreted these data to indicate that non-survivors were not capable of producing an adequate anti-inflammatory response in the alveoli early on in the ARDS disease process. A similar concept of an inverse relationship between the extent of an inflammatory response and clinical findings has been reported by Sheeran et al. (18) studying the response to respiratory syncytial virus. They measured cytokine levels in nasopharyngeal and tracheal secretions of children infected with respiratory syncytial virus. They reported that the cytokines RANTES, IL-8, and IL-10 were lower in patients with more severe clinical findings. Park et al. (19) assessed balance between pro- and anti-inflammatory agents in the BAL of ARDS patients. They reported that before the onset of ARDS, the molar ratio of TNF/IL-10 declined, indicating an imbalance toward anti-inflammation as ARDS was developing.

The design of this study did not provide for a control group. We have only an unpublished set of historical control measures of some of these biomarkers taken from 16 non-injured patients immediately following intubation. These samples were assayed identically to those in the multiple trauma patients. The non-injured baseline tracheal levels were as follows: IL-1β (median 1 pg/mL, IQR 0-4.5), TNF (no measureable amount), IL-6 (median 6 ng/mL, IQR 1-30), and IL-8 (median 224 pg/mL, IQR 72-544). When compared to these historical baseline values, the D-ALI subjects showed increases in all of the inflammatory mediators, but the increase was not to the extent of that in the N-ALI group. The subjects who developed ARDS on B-ALI were not used in the data analysis. However, the results from this group are supportive of the concept that subjects who develop ALI/ARDS have a muted inflammatory response in tracheal fluids. These differences are shown in Figures A-1, A-2, and A-3 in the Appendix.

Multiple trauma is a major risk factor for ARDS, but trauma patients are not the same as other groups of patients at risk for ALI (3,20). Calfee et al. (20) has noted that patients with lung injury related to trauma have fewer comorbidities than other at-risk groups. This makes them overall less ill and they have less mortality from ARDS than other at-risk populations. When compared to other groups of patients at risk for ARDS, trauma patients are significantly younger (1,20,21). Therefore, the utility of methods used to predict susceptibility in other at-risk groups must be verified in trauma patients. Dicker et al. (3) have reported that the consensus criteria for ARDS (9) results in lumping a disparate group of trauma patients into what is not a consistent clinical course. It appears as though trauma patients should not be lumped into other categories of ARDS patients.

Predictions of development of ALI/ARDS in patients with multiple blunt trauma by others have used multiple clinical risk factors (22), radiological assessment of the extent of pulmonary contusions (13), and the cytokine IL-8 in BAL after multiple trauma (2). Navarrete-Navarro et al. (22) studied 693 severe trauma patients (ISS ≥ 16) (48 of whom developed ARDS) to identify predictors of ARDS. The variables collected during the first 24 hours after admission most related to development of ARDS were chest trauma, femoral fracture, Acute Physiology

and Chronic Health Evaluation II score, and blood transfusions. There has been no report of the use of these variables in a follow-up study to alter patient care for those at high risk of ARDS. IL-8 has been reported to be elevated in BAL in patients who develop ALI/ARDS (2,14,21). The at-risk patients initially studied presented with severe trauma, perforated bowel, or pancreatitis, but showed no change in plasma IL-8 levels (21). A significant factor in the production of IL-8 has been reported to be systemic hypoxia (14). Raymondos et al. (2) measured IL-8 and other cytokines in BAL 2-6 hours following trauma. On the basis of BAL IL-8 levels, they stratified their patients into high risk and low risk. There was very little overlap between the IL-8 concentrations of the two groups. BAL IL-8 was a predictor of ARDS as 5 of the 8 high-risk patients developed ARDS on Day 1 following severe trauma (ISS 29), while only 2 of the 18 low-risk patients developed ARDS 1-2 weeks later. Pulmonary contusions occurring with trauma have been reported to be a significant risk factor for the development of ALI. Becher et al. (13) reported that 50% of patients with pulmonary contusions develop pneumonia or ARDS. They used customized software for radiology on computed tomography images of trauma patients on admission. When patients have pulmonary contusions involving ≥24% of their lungs, 78% of patients develop ARDS. They report that these data are available within 72 hours of the scan.

Anti-inflammatory cytokines serve to regulate the pro-inflammatory cytokines mentioned above. What is known about concentrations of anti-inflammatory cytokines in ALI is based on levels found in BAL (11,19) and in blood. CRP is a short chain pentraxin that is produced primarily by the liver and increases dramatically with systemic inflammation (acute phase reactant). It has been shown to inhibit polymorphonuclear neutrophil mediated capillary permeability in animals (23), and blood levels of CRP are positively correlated with ARDS survival (11). PTX3 is a long chain pentraxin and also an anti-inflammatory acute phase protein. PTX3 is produced peripherally by dendritic cells and monocytes (24). The cytokine IL-10 is an anti-inflammatory protein that is found in BAL of patients who have ARDS (17,19). Donnelly et al. (17) reported that low levels of the anti-inflammatory cytokine IL-10 in BAL of ARDS patients were associated with poor prognosis. All three of these anti-inflammatory mediators were lower in the tracheal aspirates (Table 2) of the patients who developed ALI after Day 1. CRP levels were very high in the plasma of the patients in this study. The plasma levels were so high that the levels of CRP measured in the tracheal aspirates could have been due to passive movement of CRP and not local production. It is not at all clear how the biomarker measured in tracheal fluids relates to the development of changes at the alveolar level of the lung.

TNF, IL-1β, and IL-6 are pro-inflammatory cytokines that have been found in the BAL of patients with a wide variety of etiologies of ARDS (6,19,25-27). The consistency of data between studies of ARDS for a role of TNF and IL-1β has led to multiple studies using TNF receptor antagonists to experimentally treat ARDS patients, but with no benefit. In addition, many studies also include monitoring of these cytokines and their soluble receptors in an attempt to understand their potential physiologic role in this disease process (19). IL-6 has been reported to increase early in the at-risk period of the development of ARDS (19). IL-6 is pleiotropic, and it is not clear whether it is functioning as a pro- or anti-inflammatory mediator. The clearly pro-inflammatory TNF and IL-1β cytokines were higher in the tracheal aspirates of the patients who did not develop ALI during the study (Table 2). The present study did not address the functionality of these cytokines, but only studied them as potential biomarkers of susceptibility for ALI/ARDS.

6.0 CONCLUSIONS

The statistically significant differences in three biomarkers in tracheal fluid between groups of trauma subjects (N-ALI vs. D-ALI) on Day 1 post injury were the key findings of this study. These results have led us to conclude that biomarkers in tracheal aspirates can be reliably measured and that they are potentially predictive of which trauma patients are at risk for developing ALI. A larger multicenter trial using only one sample site and fewer cytokines (biomarkers) would provide a larger sample size of patients necessary to determine the usefulness of this approach to predicting risk. Larger numbers of patients are needed to determine the rate of false positives and false negatives for the candidate biomarkers.

7.0 RECOMMENDATIONS

The statistically significant differences in three biomarkers on Day 1 tracheal samples between subjects who did not develop ALI and those who did after Day 1 make a compelling argument for further study. These significant findings were obtained on relatively few subjects (only six with ALI after Day 1). The following recommendations are provided to assist investigators with the next phase in avoiding problems with recruitment and simplifying the sample analysis.

1. Expand this study to all three C-STARS trauma units. This will serve to increase the number of subjects and to avoid any possible bias from using only one site.
2. Abandon the use of the TaperGuard Evac oral ETT. Ensuring placement of this tube as the initial tube in the patient was difficult. It should be eliminated, as the data collected with the port above the cuff did not provide any useful information. Collecting tracheal specimens from the large airways only would mean that any intubated patient meeting the inclusion-exclusion criteria could be enrolled.
3. Abandon the analysis of biomarkers in plasma. Restrictions on collection of dedicated samples simultaneous with collection of tracheal fluid made data from these samples hard to interpret. Also, the samples sent to the lab remained at room temperature for hours prior to processing for biomarkers. This is simply poor laboratory practice and should not be repeated.
4. Consider the utilization of telephone consent. One of the major problems with enrolling these severely injured subjects was obtaining consent. The patients could not provide consent, so it became a real challenge to find a blood relative or legal guardian. Since the only sample now being collected would be the tracheal fluid that is normally discarded, the use of consent by phone should be considered.
5. Consider expanding the target population to include patients with penetrating traumatic injury. The current study was restricted to blunt trauma only. The inclusion of penetrating trauma should be considered, but with restrictions.
6. Utilize a commercial vendor for sample analysis. The analysis of these samples required a highly skilled and experienced laboratory technician. Part of the lab tech's responsibility was to determine cell counts and differentials in the tracheal fluid. We now know that this is no longer necessary. This means that the lab tech would not have to be on call to process samples and then carry out batched assays as usual. Simple changes in

the processing of the tracheal samples need to be carried out. The analysis of fewer biomarkers could be carried out by a commercial vendor, reducing the cost of the study.

7. Ensure greater involvement in the design of the study by statisticians who have experience with predictive experiments.

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APPENDIX

Analysis of the Biomarkers IL-1β, PTX3, and TNF in Tracheal Fluid

The focus of this study was to determine if the biomarkers in the tracheal fluids of the N-ALI subjects were different from the D-ALI subjects. These analyses did not include the subjects who developed ALI/ARDS on the baseline day. The following figures show these data for the B-ALI subjects (n=5) along with the other two groups. Also included in the figures for IL-1β and TNF are unpublished results from non-injured patients taken immediately after intubation.

It is clear that these cytokines in the tracheal fluid from B-ALI subjects are also lower than the levels in the N-ALI group. It should also be noted that the historical control data indicate that there was an inflammatory response in the D-ALI group but not as great as in the N-ALI group.

Figures A-1, A-2, and A-3 are presented to demonstrate that on Day 1 those subjects who developed ARDS in the first 24 hours post injury (B-ALI) had a reduced inflammatory response relative to the N-ALI subjects. This is similar to the reduced response in the D-ALI group.

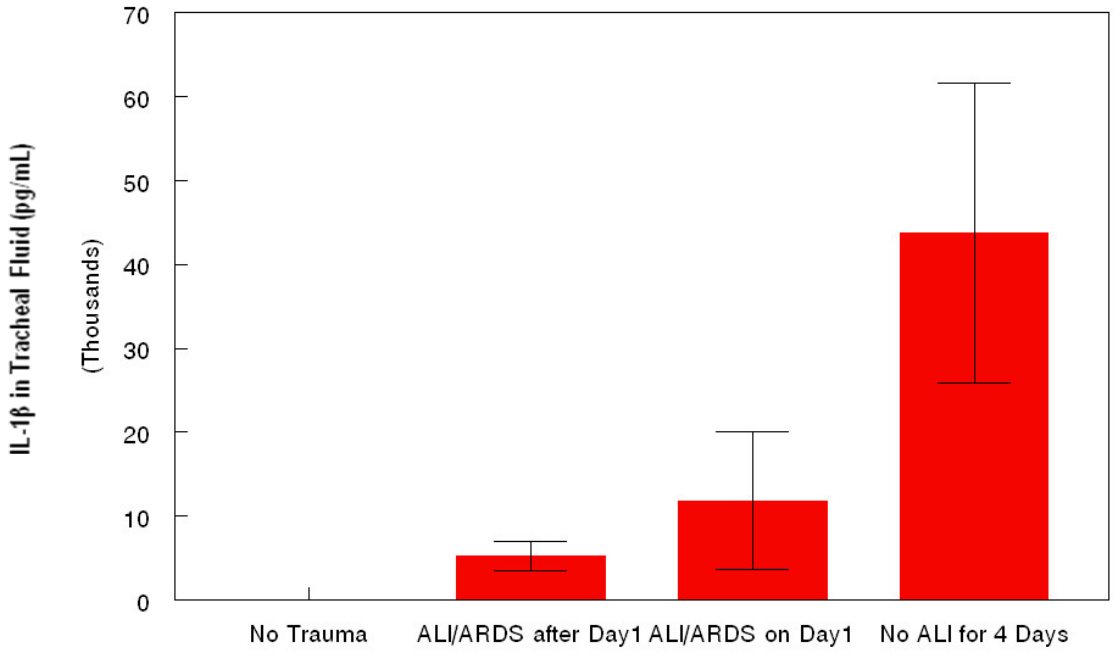

Figure A-1. Concentrations of IL-1β in Tracheal Fluid from the Baseline Sample on Day 1 Following Severe Blunt Trauma for Three Groups of Subjects: D-ALI, B-ALI, and N-ALI. *The D-ALI group (n=6) had significantly less IL-1β ($p<0.05$) than the N-ALI subjects (n=13). Historical control data are also shown for non-injured subjects (n=16) from samples collected immediately after intubation. Significance was determined by Mann-Whitney test.*

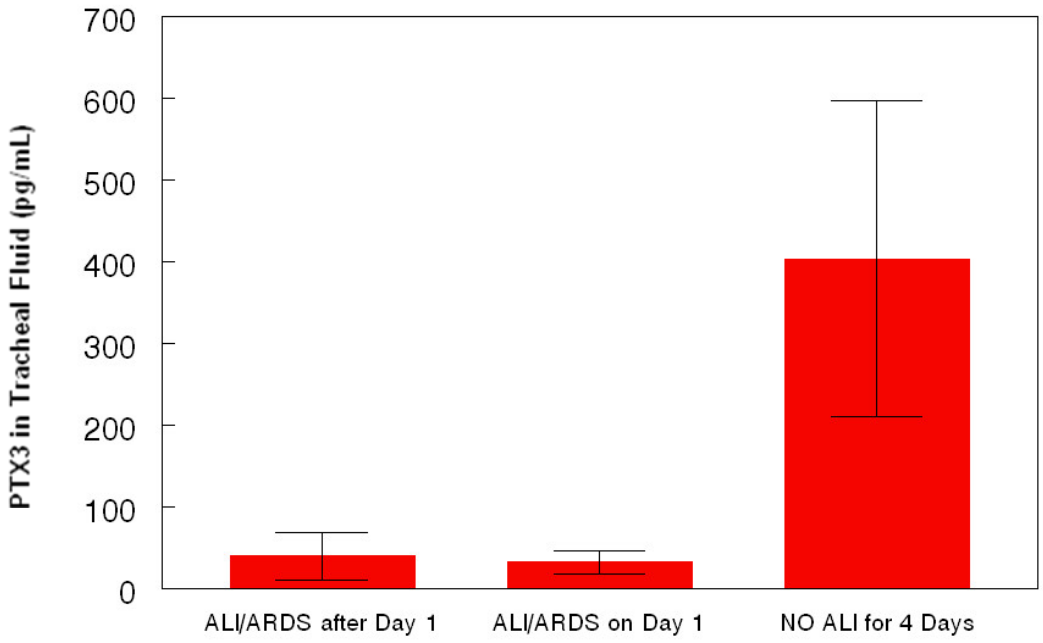

Figure A-2. Concentrations of PTX3 in Tracheal Fluid from the Baseline Sample on Day 1 Following Severe Blunt Trauma for Three Groups of Subjects: D-ALI, B-ALI, and N-ALI. *The D-ALI group (n=6) had significantly less PTX3 ($p<0.05$) than the N-ALI subjects (n=13). Significance was determined by Mann-Whitney test.*

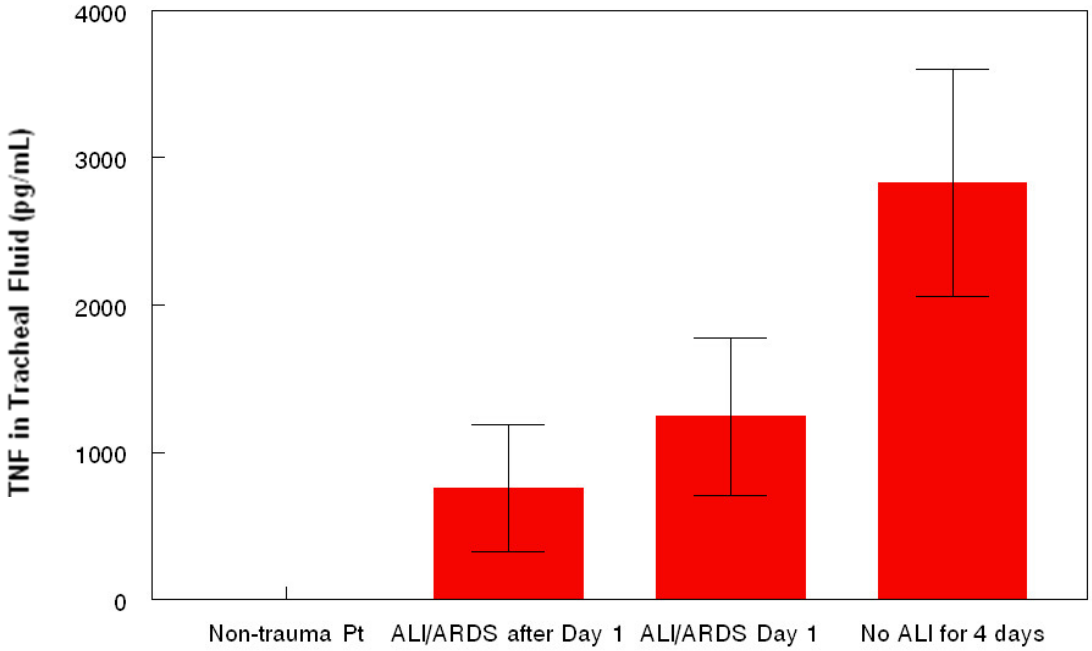

Figure A-3. Concentrations of TNF in Tracheal Fluid from the Baseline Sample on Day 1 Following Severe Blunt Trauma for Three Groups of Subjects: D-ALI, B-ALI, and N-ALI. *The D-ALI group (n=6) had significantly less TNF ($p<0.05$) than the N-ALI subjects (n=13). Historical control data are also shown for non-injured subjects (n=16) from samples collected immediately after intubation. Significance was determined by Mann-Whitney test.*

LIST OF ABBREVIATIONS AND ACRONYMS

ALI	acute lung injury
B-ALI	group of subjects who developed ALI or ARDS on baseline day, Day 1
D-ALI	group of subjects who developed ALI delayed to after Day 1
N-ALI	group of subjects who did not develop ALI for the 4 days of the study
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
C5a	activated complement 5
CRP	C-reactive protein
ELISA	enzyme-linked immunosorbent assay
ETT	endotracheal tube
IL	interleukin
ISS	Injury Severity Score
PTX3	pentraxin 3
PMN	polymorphonuclear neutrophils
TNF	tumor necrosis factor